BIOETHANOL PRODUCTION IN *PICHIA STIPITIS*: KINETIC STUDIES IN A CHEMOSTAT AND DEVELOPMENT OF GENETIC TOOLS TO OVERCOME KINETIC LIMITATIONS

SHRADDHA MAITRA

DEPARTMENT OF BIOCHEMICAL ENGINEERING AND BIOTECHNOLOGY

INDIAN INSTITUTE OF TECHNOLOGY DELHI

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by

SHRADDHA MAITRA

Department of Biochemical Engineering and Biotechnology

Submitted

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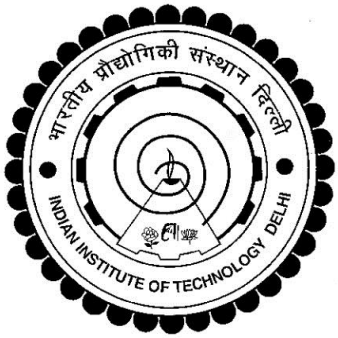

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CERTIFICATE

This is to certify that the thesis entitled **"Bioethanol production by *Pichia stipitis*: kinetic studies in a chemostat and development of genetic tools to overcome kinetic limitations"** being submitted by **Ms Shraddha Maitra** is worthy of consideration for the award of the degree of **Doctor of Philosophy**. The thesis has been prepared by her under my supervision and guidance in conformity with the rules and regulations of Indian Institute of Technology Delhi and is a record of the original bonafide research work. The results presented in this thesis have not been submitted in part or full to any other universities or institutes for the award of any other degree or diploma.

Date:

Prof. Atul Narang
Department of Biochemical Engineering & Biotechnology
Indian Institute of Technology Delhi
Hauz Khas, New Delhi-110016

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ABSTRACT

Dwindling resources of fossil fuels, environmental concern and socio-political issues have triggered the search for alternative sources of energy. Biofuels may provide the solution to this problem. For efficient production of bioethanol from lignocellulosic hydrolysate, the organism should ferment a variety of sugars (hexoses and pentoses) with high ethanol yield and productivity. The yeast *Scheffersomyces stipitis* (earlier known as *Pichia stipitis*) possesses these properties. However, it has not found widespread use primarily because early studies showed that *S. stipitis* grows but does not ferment under aerobic conditions, and ferments but does not grow under anaerobic conditions. Thus, both growth and fermentation occur only in a narrow range of small but non-zero dissolved oxygen levels. This has led to the conclusion that ethanol production is not robust and cannot be scaled up. However, lack of robustness cannot be established by showing that ethanol is produced in a narrow range of oxygen levels. It is necessary to show that such dissolved oxygen levels are obtained only in a narrow range of operating conditions.

In this work, we have devised such a method for quantifying the robustness of ethanol production with respect to the operating conditions. Our approach is based on the principles of growth on a mixture of two complementary substrates. We show that when the chemostat culture of *S. stipitis* is fed with progressively higher glucose feed concentrations (s_f) at a fixed dilution rate ($D = 0.1$ h^{-1}) and oxygen mass transfer coefficient ($k_l a = 50$ h^{-1}), three definite growth regimes are obtained. At low s_f, the culture is carbon limited – dissolved oxygen is at saturating levels, and no ethanol is produced. At high s_f, the culture is oxygen limited – ethanol is produced but excess glucose is observed in the effluent stream. At intermediate s_f, both carbon and oxygen are limiting and ethanol is produced without any loss of glucose. It is plausible to say that ethanol production is robust if the width of this

regime is large. We also developed a simple unstructured mathematical model that predicts the boundaries of the dual-limited regime.

The model predictions agree with the data, but they imply that the boundaries vary with D and $k_l a$. Thus, we were led to perform the foregoing experiment at additional D and $k_l a$. We find that the boundaries predicted by the model are consistent with the data obtained at all D and $k_l a$. Moreover, we show that if the biomass, ethanol, dissolved oxygen and residual glucose concentrations, are scaled appropriately and plotted against the dimensionless parameter, $\rho = Ds_f / k_l a \cdot c_o^*$, all the data obtained at various D, $k_l a$ and s_f, collapses into a single curve. The existence of the dimensionless plot facilitates strain comparison without significant acquisition of data.

Finally, the inaccessibility of plasmid vectors for *S. stipitis* prevented us from modifying the strain for overcoming its kinetic limitations. Therefore, we developed plasmids specific to *S. stipitis* which can be used for both wild-type and auxotrophic strains. Interestingly, *S. stipitis* exhibited improved growth and glucose consumption rates upon over-expression of the sugar transporter SUT4. This indicates limitations at the level of sugar uptake.

सार

जीवाश्म ईंधन के सीमित भण्डार, पर्यावरणीय चिंता और सामाजिक-राजनीतिक मुद्दों के करण ऊर्जा के वैकल्पिक स्रोतों की खोज शुरू कर दी है। बायोएथनोल इस समस्या का समाधान प्रदान कर सकते हैं। लिग्नोसेल्युलोसिक हाइड्रोलाइजेट से बायोएथनोल के कुशल उत्पादन के लिए, जीव को उच्च इथेनॉल उपज और उत्पादकता के साथ विभिन्न शक्कर (हेक्सोज़ और पेंटोज़) को विक्षोभ करना चाहिए। खमीर *स्केफ्फेर्सोमिसेस स्टिपिटिस* (जिसे पहले *पिकया स्टेपिटिस* के नाम से जाना जाता है) के पास यह गुण है। हालांकि, इसे मुख्य रूप से व्यापक रूप से उपयोग नहीं मिला है क्योंकि प्रारंभिक अध्ययनों से पता चला है कि *एस स्टेपिटिस* एरोबिक स्थितियों बढ़ता है लेकिन विक्षोभ नहीं करता है लेकिन एनारोबिक स्थितियों के तहत विक्षोभ करता है लेकिन नहीं बढ़ता है। इस प्रकार, विकास और विक्षोभ दोनों केवल छोटे लेकिन गैर-शून्य ऑक्सीजन के स्तर की एक संकीर्ण सीमा में होते हैं। इसलिये निष्कर्ष निकाला है कि इथेनॉल उत्पादन मजबूत नहीं है और इसे स्केल अप नहीं किया जा सकता है। हालांकि, मजबूती की कमी को यह दिखाकर स्थापित नहीं किया जा सकता है कि इथेनॉल ऑक्सीजन के स्तर की एक संकीर्ण श्रृंखला में उत्पादित होता है। यह दिखाने के लिए आवश्यक है कि इस तरह के विघटित ऑक्सीजन स्तर केवल परिचालन स्थितियों की एक संकीर्ण सीमा में प्राप्त किए जाते हैं।

इस काम में, हमने परिचालन स्थितियों के संबंध में इथेनॉल उत्पादन की मजबूती को मापने के लिए ऐसी विधि तैयार की है। हमारा दृष्टिकोण दो पूरक सबस्ट्रेट्स के मिश्रण पर विकास के सिद्धांतों पर आधारित है। हम दिखाते हैं कि जब *एस स्टेपिटिस* की कीमोस्टाट को निश्चित रूप से उच्च ग्लूकोज फ़ीड सांद्रता (s_f) के साथ एक निश्चित डयलूशन रेट ($D = 0.1\ \mathrm{h}^{-1}$) और ऑक्सीजन द्रव्यमान स्थानांतरण गुणांक ($k_l a = 50\ \mathrm{h}^{-1}$) पर खिलाया जाता है, तीन निश्चित विकास शासन प्राप्त किए जाते हैं। कम s_f पर, कार्बन सीमित है - ऑक्सीजन संतृप्त स्तर

पर है, और कोई इथेनॉल का उत्पादन नहीं होता है। उच्च s_f पर, ऑक्सीजन सीमित है - इथेनॉल का उत्पादन होता है लेकिन अवशिष्ट प्रवाह में अतिरिक्त ग्लूकोज पाया जाता है। इंटरमीडिएट s_f में, दोनों कार्बन और ऑक्सीजन सीमित हैं और इथेनॉल ग्लूकोज के किसी भी नुकसान के बिना उत्पादित होता है। यह कहना उचित है कि अगर इस क्षेत्र की चौड़ाई बड़ी है तो इथेनॉल उत्पादन मजबूत है। हमने एक सरल असंगठित गणितीय मॉडल भी विकसित किया जो दोहरी-सीमित क्षेत्र की सीमाओं की भविष्यवाणी करता है।

मॉडल भविष्यवाणियां डेटा से सहमत हैं, लेकिन वे दर्शाती हैं कि सीमाएं D और $k_l a$ के साथ भिन्न होती हैं। इस प्रकार, हमने अतिरिक्त D और $k_l a$ पर पूर्वगामी प्रयोग को पूरा किया। हम पाते हैं कि मॉडल द्वारा भविष्यवाणी की गई सीमाएं सभी D और $k_l a$ में प्राप्त डेटा के अनुरूप हैं। इसके अलावा, हम दिखाते हैं कि यदि बायोमास, इथेनॉल, ऑक्सीजन और अवशिष्ट ग्लूकोज सांद्रता को उचित रूप से स्केल किया जाता है और आयामरहित पैरामीटर के खिलाफ प्लॉट किया जाता है, तो प्राप्त सभी डेटा विभिन्नD, $k_l a$ और s_f पर, एक वक्र में गिर जाता है। आयामरहित पैरामीटर डेटा अर्जन और जीव तुलना की सुविधा प्रदान करता है।

अंत में, *एस स्टेपिटिस* के प्लास्मिड वैक्टरों की कमी ने हमें जीव को संशोधित करने से रोका। इसलिए, हमने *एस स्टेपिटिस* के लिए विशिष्ट प्लास्मिड् विकसित किए हैं जिनका उपयोग जंगली प्रकार और ऑक्सोट्रोफिक उपभेदों दोनों के लिए किया जा सकता है। दिलचस्प बात यह है कि *एस स्टेपाइटिस* ने चीनी ट्रांसपोर्टर SUT4 की अति अभिव्यक्ति पर वृद्धि करने से ग्लूकोज खपत दरों में सुधार आया है । यह चीनी के अपटेक स्तर पर कमी को इंगित करता है।

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LIST OF ABBREVIATIONS

CDW	Cell dry weight
C-limited	Carbon limited
C mol	Carbon mol
C-source	Carbon source
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
GC	Gas chromatography
HPLC	High performance liquid chromatography
N-limited	Nitrogen limited
N-source	Nitrogen source
OD	Cell optical density
O-limited	Oxygen limited
YIp	Yeast integrative plasmid
YPD medium	Yeast extract Peptone Dextrose medium
YRp	Yeast replicative plasmid

LIST OF SYMBOLS

c_o^*	Solubility of oxygen in the medium at the experimental conditions (g l^{-1})
c_o	Dissolved oxygen concentration (g l^{-1})
D	Dilution rate (h^{-1})
$k_l a$	Mass transfer coefficient (h^{-1})
p	Product (Ethanol) concentration (g l^{-1})
r_O	Specific oxygen consumption rate in C-limited regime (g g^{-1} h^{-1})
r_O'	Specific oxygen consumption rate in O-limited regime (g g^{-1} h^{-1})
r_p	Specific ethanol productivity in C-limited regime (g g^{-1} h^{-1})
r_p'	Specific ethanol productivity in O-limited regime (g g^{-1} h^{-1})
r_s	Specific glucose consumption rate in C-limited regime (g g^{-1} h^{-1})
r_s'	Specific glucose consumption rate in O-limited regime (g g^{-1} h^{-1})
s	Residual glucose concentration (g l^{-1})
$\underline{s}_f$	Lower boundary of transition regime (g l^{-1})
$\overline{s}_f$	Upper boundary of transition regime (g l^{-1})
s_f	Feed glucose concentration (g l^{-1})
t	Time (h)
x	Biomass concentration (gdw l^{-1})
Y_{op}	Yield of ethanol on oxygen in C-limited regime (g g^{-1})

Y'_{op}	Yield of ethanol on oxygen in O-limited regime (g g^{-1})
Y_{ox}	Yield of biomass on oxygen in C-limited regime (g g^{-1})
Y'_{ox}	Yield of biomass on oxygen in O-limited regime (g g^{-1})
Y_{sc}	CO_2 produced per gram of glucose in C-limited regime (g g^{-1})
Y'_{sc}	CO_2 produced per gram of glucose in O-limited regime (g g^{-1})
Y_{so}	Oxygen consumed per gram of glucose in C-limited regime (g g^{-1})
Y'_{so}	Oxygen consumed per gram of glucose in O-limited regime (g g^{-1})
Y_{sp}	Product (Ethanol) yield in C-limited regime (g g^{-1})
Y'_{sp}	Product (Ethanol) yield in O-limited regime (g g^{-1})
Y_{sx}	Biomass yield in C-limited regime (g g^{-1})
Y'_{sx}	Biomass yield in O-limited regime (g g^{-1})
μ	Specific growth rate (h^{-1})
μ_m	Maximum specific growth rate (h^{-1})
π	Normalised dimensionless product (ethanol)
ρ	Dimensionless ratio of operating parameters
σ	Normalised dimensionless residual glucose
χ	Normalised dimensionless biomass
ω	Normalised dimensionless dissolved oxygen